SUPPLEMENTAL

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Supplemental Materials And Methods

Antibodies and reagents. TR3 and Sp1 antibodies were purchased from Imgenex (San Diego, CA) and Upstate (Temecula, CA), respectively. Flag and β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Bcl-2 and p300 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and all remaining antibodies were purchased from Cell Signaling Technology (Beverly, MA). DIM-C-pPhOH was synthesized in this laboratory as previously described (14), and the identity and purity (>98%) were confirmed by gas chromatography-mass spectrometry. Reporter lysis buffer and luciferase reagent were supplied by Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA).

Western blot analysis and immunoprecipitation. Cells (2×10^5) were plated in six-well plates in DMEM media containing 10% FBS for 16 hr and then treated. Cellular lysates were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride, 5 μL/ml Protease inhibitor cocktail (Sigma-Aldrich) and 1% NP-40. The cells were disrupted and extracted at 4°C for 30 min. After centrifugation at 15,000 x *g* for 15 min, the supernatant was obtained as the cell lysate. Nuclear extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol. Protein concentrations were measured using the Bio-Rad protein assay. Aliquots of cellular proteins were electrophoresed on 8-12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The

membrane was allowed to react with a specific antibody and detection of specific proteins was carried out by enhanced chemiluminescence (PerkinElmer). Immunoprecipitation of TR3 and p53 was carried as described (Lee *et al.*, 2010b) Loading differences were normalized using a polyclonal β-actin antibody.

Quantitative real-time PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and cDNA was prepared from the total RNA using Reverse Transcription System (Promega). Each PCR was carried out in triplicate in a 20-μL volume using SYBR Green Mastermix (Applied Biosystems, Foster City, CA) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min in the Applied Biosystems 7900HT Fast Real-time PCR System. The ABI Dissociation Curves software was used following a brief thermal protocol (95°C for 15 sec and 60°C for 15 sec, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein (TBP). The sequences of the primers used for real-time PCR were as follows: sestrin-2 sense 5'-CAA GCT CGG AAT TAA TGT GCC-3', antisense 5'-CTC ACA CCA TTA AGC ATG GAG-3'; survivin sense 5'-CAG ATT TGA ATC GCG GGA CCC-3', antisense 5'-CCA AGT CTG GCT CGT TCT CAG-3'; and TBP sense 5'-TGC ACA GGA GCC AAG AGT GAA-3', antisense 5'-CAC ATC ACA GCT CCC CAC CA-3'. The PCR primers for TR3 were purchased from Qiagen.

Cell proliferation assay. Cells (1×10^5 per well) were plated in 12-well plates and allowed to attach for 16 hr. The medium was then changed to DMEM medium containing 2.5% FBS, and either vehicle (DMSO) or different concentrations of the compound were added. Fresh medium and compounds were added every 48 hr, and

cells were then trypsinized and counted after 24, 48, and 72 hr using a Coulter Z1 cell counter (Beckman Coulter Inc., Fullerton, CA).

Reporter gene assay. Cells (1 x 10^5 cells/well) were plated in 12-well plates in DMEM media supplemented with 10% FBS. After 16 hr, various amounts of DNA were transfected using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer's protocol. After transfection for 5 hr, the transfection mix was replaced with complete media containing either vehicle (DMSO) or different concentrations of the compound for 18 hr. Cells were then lysed with 150 μL of 1x reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-galactosidase assays. A multifunctional microplate reader (FLUOstar OPTIMA) was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity. The corresponding empty vector was used as a control.

DNA-binding assay. GC-rich DNA binding of Sp1 and p300 was measured using the Universal EZ-TFA transcription factor assay Chemiluminescent kit (Upstate Biotechnology, Inc) according to the manufacturer's protocol. After treatment with the compound or siRNAs, nuclear extracts were prepared and incubated with a biotinylated double-stranded oligouncleotide containing Sp1 consensus sequence (capture probe) in streptavidin-coated plate for 1~2 hr at room temperature. To ensure that the DNA binding is in a sequence specific manner, an unlabeled competitor oligonucleotide containing the identical consensus sequence as the capture probe was used. A negative control without the capture probe was also used in each assay. The DNA-protein complex was immobilized on the plate, and the unbound materials were washed away. The bound protein was then incubated with a rabbit anti-Sp1 antibody or a rabbit

anti-p300 antibody for 1 hr at room temperature followed by incubation with rabbit IgG-horseradish peroxidase (HRP) conjugated secondary antibody for 30 min. Luminescence of HRP was detected using a multifunctional microplate reader (FLUOstar OPTIMA).

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay. Tissue sections were deparaffinized in xylene and then treated with a graded series of alcohol [100, 95, 85, 70, and 50% ethanol (v/v) in double-distilled water] and rehydrated in PBS (pH 7.5). Tissues were then treated with proteinase K solution for permeabilization and then refixed with 4% paraformaldehyde solution. Slides were then treated with recombinant terminal deoxynucleotidyl transferase reaction mix and incubated at 37°C for 1 hr. Reaction was terminated by immersing the slides in 2x SSC solutions for 15 min at room temperature and slides were washed three times with PBS. After washing, slides were mounted and images were collected and analyzed using a Zeiss Axioplan2 microscope (Carl Zeiss).

In Vivo **Experiments**

Orthotopic implantation of A549 NSCLC in lung of Nu/Nu mice and treatment. Female, 5-6 weeks old, athymic nu/nu mice (Harlan Laboratories, Indianapolis, IN) were housed and maintained under pathogen-free condition in an AAALAC-accredited facility. The animals were fed standard feed and water *ad libitum* and housed in standard cages. All procedures were carried out in accordance with requirements of the Institutional Animal Care and Use Committee, Florida A&M University. To induce tumor growth in the lungs, A549 NSCLC cells were harvested from subconfluent cultures using 0.25%

trypsin containing 0.02% EDTA. The cells were washed and suspended in phosphate buffered saline (PBS) and checked for viability, ensuring that single cell suspensions of viability >90% were used for the subsequent implantation.

Mice were anaesthetized by brief exposure to halothane and laid in the right lateral decubitus position. A 5 mm transverse skin incision was made approximately 5 mm inferior to the scapula of the left chest to expose the lung through the intercostal muscles. A 28-gauge hypodermic needle was used to inject A549 cell inoculum (1 x 10^6 cells per mouse) dispersed in a final volume of 100 μL of PBS (pH 7.4) through the sixth intercostal space into the left lung. The needle was inserted to a depth of about 3 mm and quickly removed. The skin incision was closed with surgical skin clips, and the animals observed for up to 60 minutes for full recovery.

The animals were randomized into two groups 24 hr post inoculation; a control (*n* = 10) that received 160 μL of corn oil and a second group (*n* = 10) that was given DIM-C-pPhOH (30 mg/kg/d) in corn oil by oral gavage for 5 consecutive days. Treatment was started on day 3 post tumor cell injection and continued up to day 28. On day 28, all animals were sacrificed by exposure to a lethal dose of halothane and lungs excised, washed in PBS and assessed for tumor regression. Bilateral lungs as well as other organs (heart, liver, kidneys, and spleen) were removed, and either fixed in 10% formalin and embedded in paraffin or snap frozen in liquid nitrogen and stored at -80°C. Histologic sections were made from the lung tissues and stained with hematoxylin and eosin (H&E) for observation under a microscope.

Intravenous injection of A549 NSCLC in Nu/Nu mice and treatment. Female, 5-6 weeks old, athymic nu/nu mice were raised under pathogen-free condition in an

AAALAC-accredited facility. The animals were fed standard feed and water *ad libitum* and housed in standard cages. All procedures were carried out in accordance with requirements of the Institutional Animal Care and Use Committee, Florida A&M University.

A549 NSCLC cells harvested from subconfluent cultures were washed and dispersed as single cell suspensions in PBS (pH 7.4) of viability >90%. Suspensions of A549 cells (2×10^6 per mouse) in a final volume of 100 μL PBS were injected into female athymic nude mice by the intravenous route through the tail vein to induce pulmonary metastasis. Animals were kept and observed for 14 days until tumor growth in lungs was confirmed by sacrificing one animal and observing the lung morphology. The animals were randomized into two groups 24 hr post inoculation; a control (n = 10) that received 160 μL of corn oil and a second group (n = 10) that was given DIM-C-pPhOH (30 mg/kg/d) in corn oil by oral gavage for 5 consecutive days. Treatment was commenced on day 15 post tumor injection and continued for 28 days. The lungs and other major organs were removed for histological studies and assessment of tumor regression.

TUNEL assessment of apoptosis of lung tumor tissues. Apoptotic detection of orthotopic and metastatic lung tissue sections were carried out following manufacturer's instruction using the DeadEnd Colorimetric Apoptosis Detection System, Promega (Madison, WI). Paraffin-embedded sections were permeabilized with xylene and pre-equilibrated with equilibration buffer. DNA strand breaks were labeled with biotinylated nucleotide mix (60 min at 37°C) and the reaction was stopped by immersing slides in 2X SSC (15 min at room temperature). Slides were washed in PBS and blocked with

hydrogen peroxide and again washed in PBS. Streptavidin HRP diluted in PBS was added to sections (30 min at room temperature) and then washed in PBS. DAB chromogen was applied to sections and reddish-brown staining allowed to develop (approximately 10 min). Slides were then rinsed several times in deionized water, mounted using permanent mounting medium, and observed under a light microscope.

Supplemental Table 1. TR3 expression in human lung cancer tissues.

TR3[1)]	ADC, n (%)	CS, n (%)	LCC, n (%)	SQC, n (%)	Total, n
Absent	-	-	-	-	-
Low	2 (13.3)	-	-	-	2
Moderate	11 (73.3)	-	-	9 (24.3)	20
High	2 (13.3)	2 (100)	5 (100)	28 (75.7)	37
Total, n (%)	15 (100)	2 (100)	5 (100)	37 (100)	59

[1)] Immunostaining intensity was scored as absent, low, moderate, or high. There was no specific staining when secondary antibody was used alone as a negative control. ADC, adenocarcinoma; CS, carcinosarcoma; LCC, large cell carcinoma; SQC, squamous cell carcinoma.

Supplemental Table 2. Correlation between clinical outcome and TR3 immunostaining in NSCLC.

Variable	Case (n)	TR3 staining		χ^2 (*P*)	Odds ratio (95% CI)
		≤ Moderate	High		
Gender					
Male	47	14	33	3.08 (NS)	0.2 (0.1-1.0)
Female	11	7	4		
Age (year)					
≤65	38	15	23	0.18 (NS)	1.5 (0.5-4.8)
≥65	20	6	14		
pT factor					
T_{1-2}	49	18	31	0.03 (NS)	1.2 (0.3-5.2)
T_{3-4}	9	3	6		
pN factor					
Negative	36	14	22	0.07 (NS)	1.4 (0.5-4.2)
Positive	22	7	15		
Cancer type					
ADC	14	12	2	16.86 (*P*<0.0001*)	23.3 (4.4-123.5)
Non-ADC	44	9	35		
Cancer stage					
I	30	12	18	0.12 (NS)	1.4 (0.5-4.1)
II-IV	28	9	19		

Abbreviations: ADC, adenocarcinoma; non-ADC, carcinosarcoma, large cell carcinoma, and squamous cell carcinoma; CI, confidence interval; NS, no significance.
* Statistically significant (Chi-square test).

Supplementary Table 3. Tumor regression data for the orthotopic mouse model of lung cancer treated with DIM-C-pPhOH.

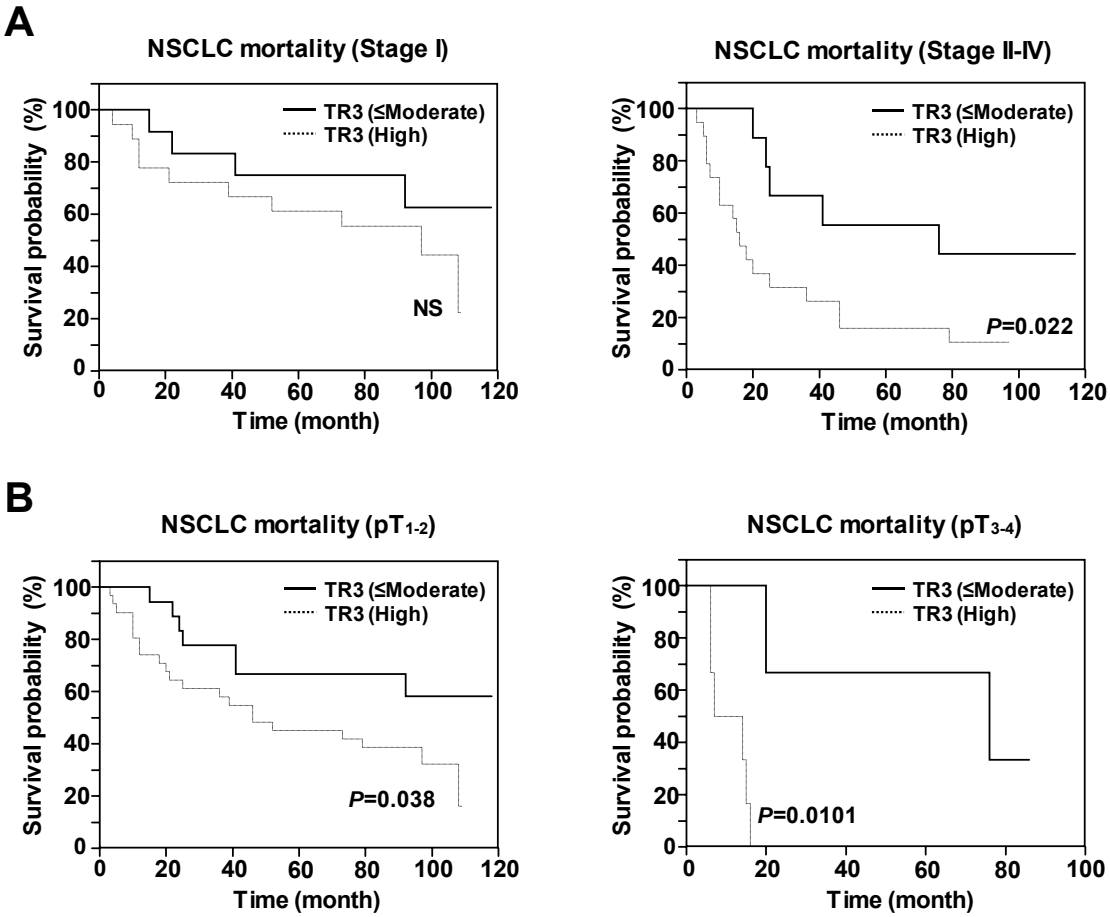
Group	Tumor incidence	Body weight (g)	Lung weight (mg)	Tumor weight (mg)	Tumor burden (TW/BW)[†]	Tumor volume (mm^3)
Control	10/10	24.30±0.76	249.88±27.39	21.78±4.90	1.05±0.08	11.54±4.96
DIM-C-pPhOH (30 mg/kg/d)	10/10	24.23±0.50	262.29±38.58	6.04±3.73[#]	0.27±0.12[#]	5.01±4.11*

[†]Tumor weight/Body weight
*P<0.05 and [#]P<0.001 vs Control

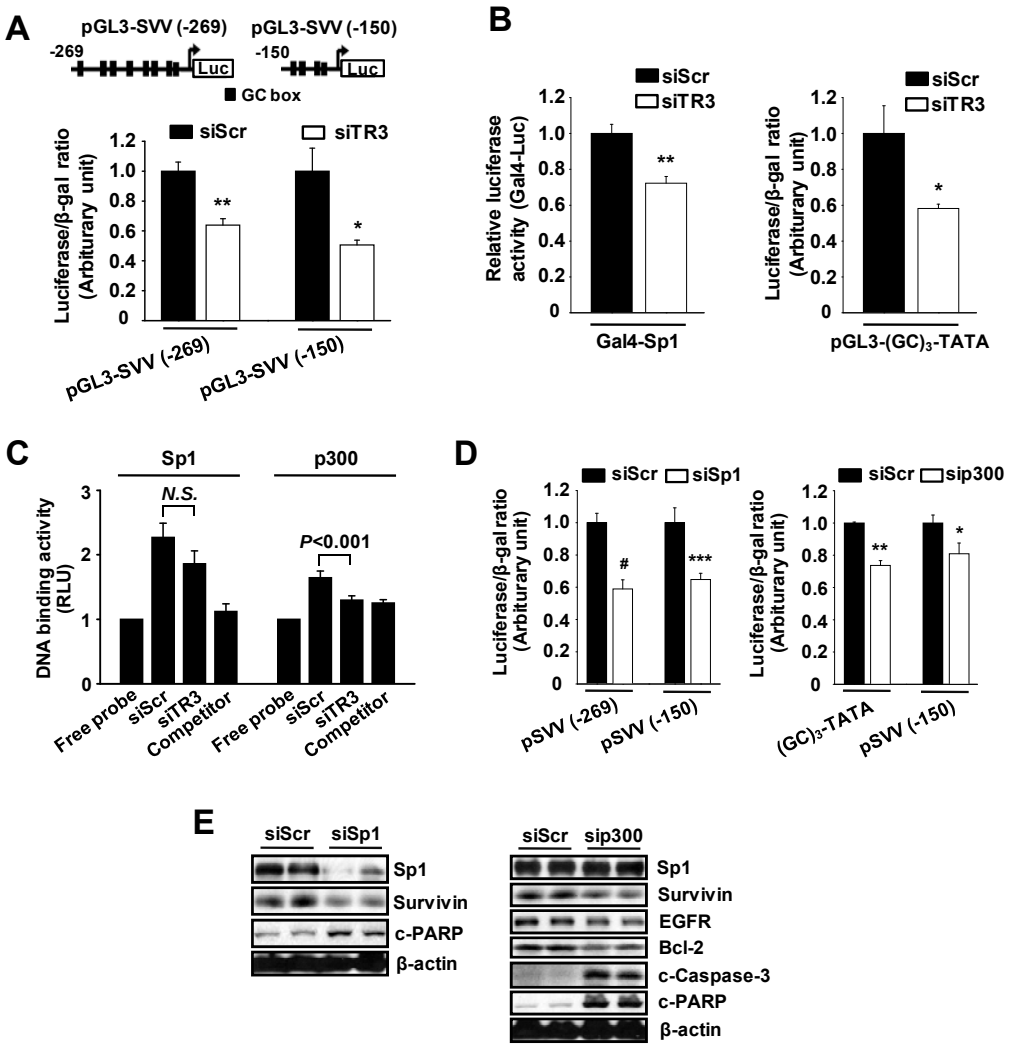
Supplemental Table 4. Tumor regression data for the metastatic mouse model of lung cancer treated with DIM-C-pPhOH.

Group	Body weight (g)	Lung weight (mg)	Tumor weight (mg)	Tumor burden (TW/BW)†	Number of nodules	Tumor volume (mm^3)
Control	25.23±0.44	227.18±17.18	99.93±16.70	0.42±0.08	5.0±0.71	164.36±46.06
DIM-C-pPhOH (30 mg/kg/d)	24.53±0.53	197.67±22.01	57.60±3.96*	0.24±0.01*	3.0±1.41*	59.80±7.91*

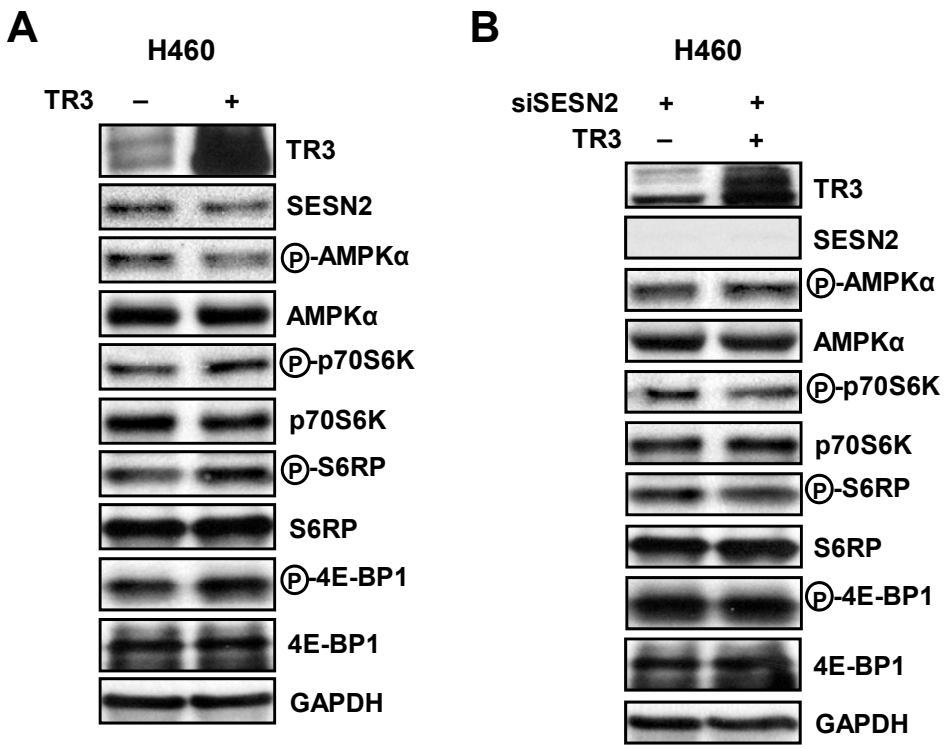
†Tumor weight/Body weight
*P<0.05 vs Control



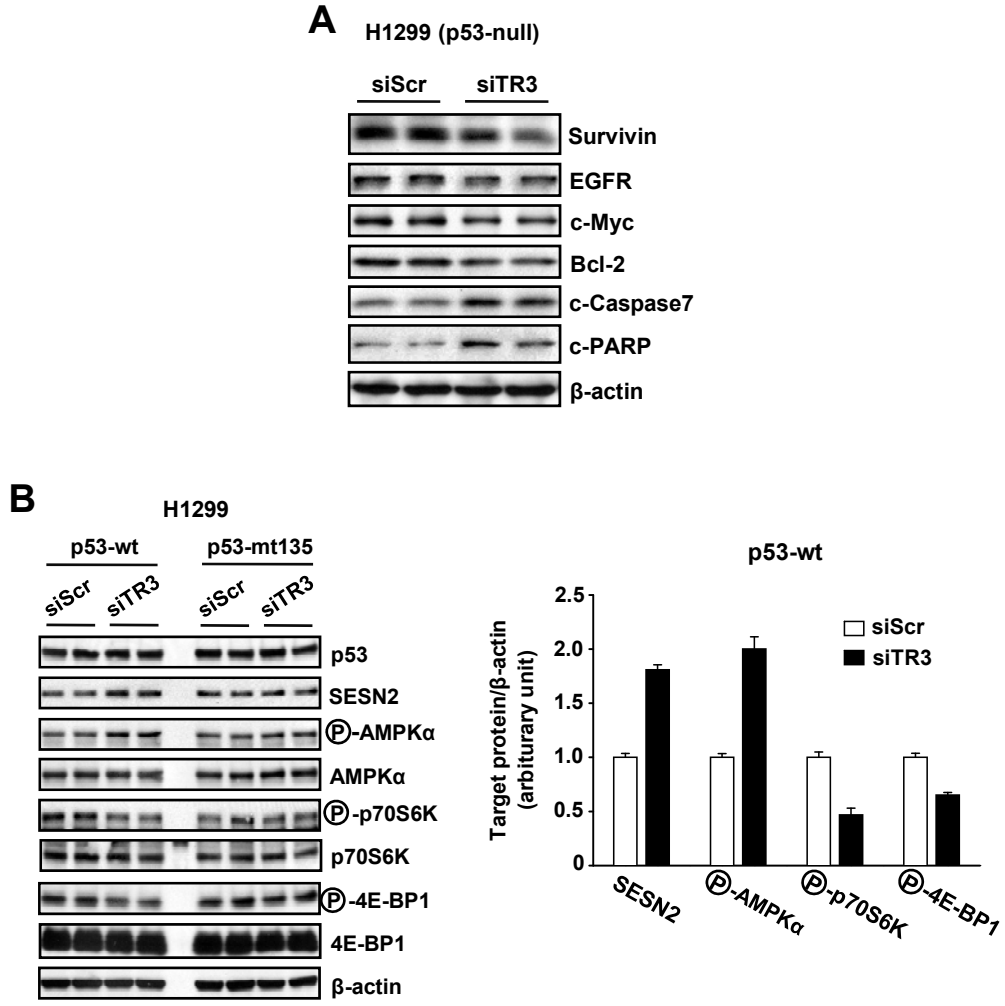
Supplemental Figure 1. Association of TR3 overexpression with poor clinical outcome for NSCLC patients. (A) Left, Kaplan-Meier survival analysis among group 1 patients with stage I NSCLC (n=30) according to TR3 expression. Right, Kaplan-Meier survival analysis among group 2 patients with stage II-IV NSCLC (n=28) according to TR3 expression. (B) Left, Kaplan-Meier survival analysis among group 1 patients with pT_{1-2} NSCLC (n=49) according to TR3 expression. Right, Kaplan-Meier survival analysis among group 2 patients with pT_{3-4} NSCLC (n=9) according to TR3 expression.



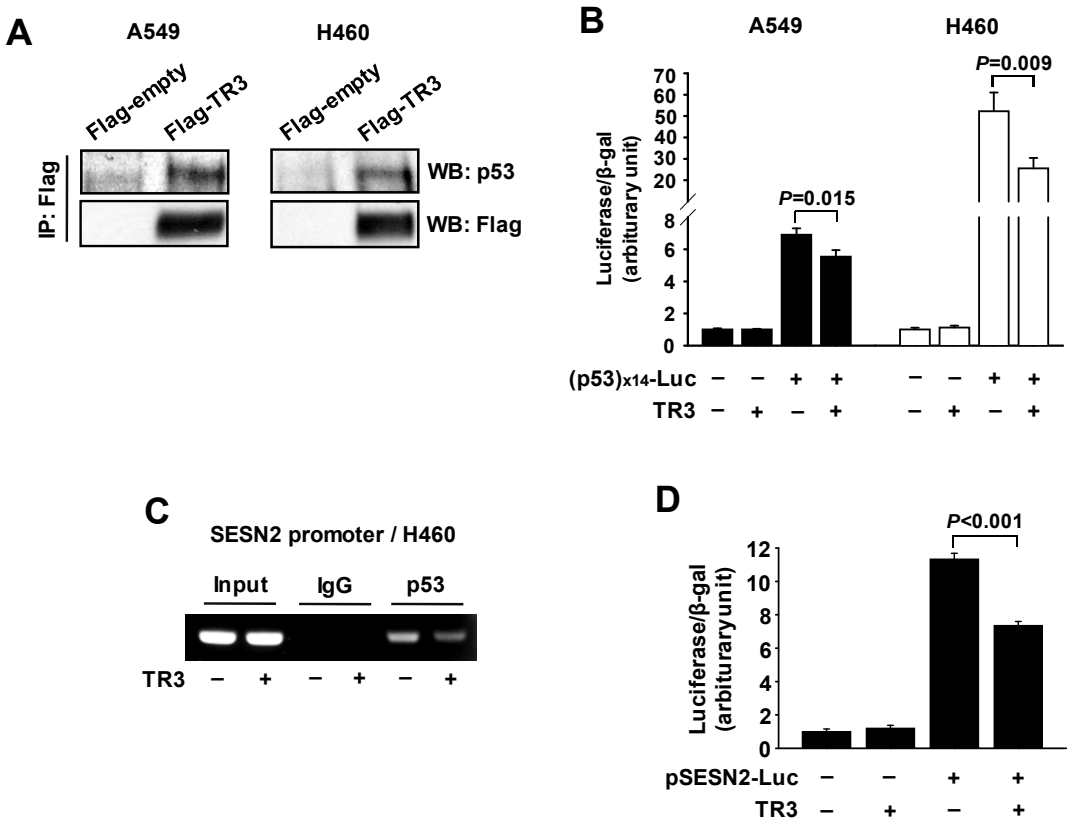
Supplemental Figure 2. Knockdown of TR3 induces apoptosis in p53 wild-type and p53-null lung cancer cells through inhibition of p300/Sp1 axis. (A and B) H460 (p53 wild-type) cells were cotransfected with each siRNA and luciferase reporter plasmid as indicated, and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control and the results are presented as means with SD of 3 experiments. **P*<0.01 and ***P*<0.005 vs siScr. (C) Effect of siTR3 on Sp1 or p300 binding to GC-rich Sp1 consensus sequence. H460 cells were transfected with either siScr or siTR3 for 60 hr, and nuclear extracts were tested for DNA binding activity as described in the Materials and Methods. An unbiotinylated oligonucleotide containing the identical consensus sequence as the capture probe was used as a competitor. The data are presented as means with SD of 3 experiments. (D) H460 cells were cotransfected with each siRNA and luciferase reporter plasmids as indicated, and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control, and the results are presented as means with SD of 3 experiments. **P*<0.05 and ***P*<0.01, ****P*<0.005, $^{\#}$*P*<0.001 vs siScr. (E) H460 cells were transfected with an indicated siRNA for 72 hours, and whole cell lysates were analyzed by western blot analysis. β-Actin was used as a loading control, and the experiment was repeated three times with similar results.



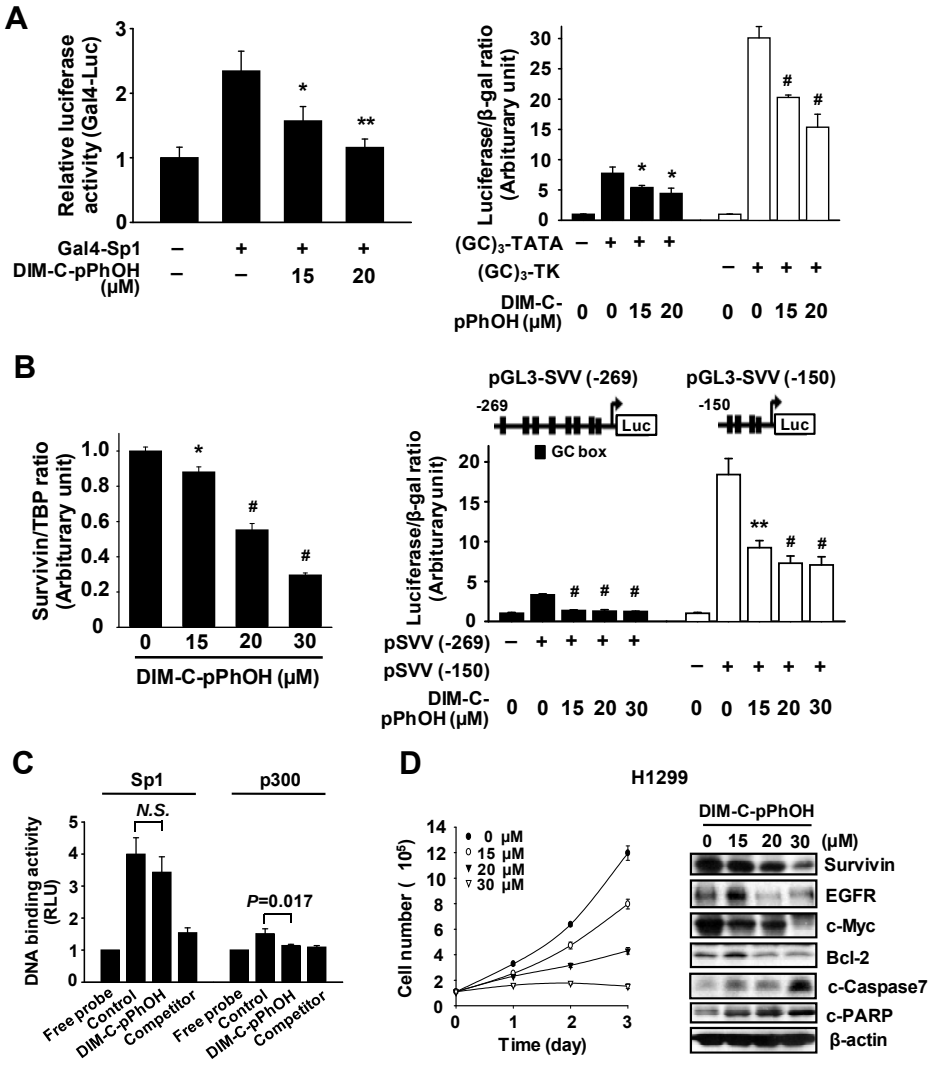
Supplemental Figure 3. Effect of TR3 overexpression on mTORC1 signaling in H460 cells. (A) Cells were transfected with adenovirus expressing TR3 or vector only (10 MOI) for 6 hr. At 24 hr after transfection, the cells were incubated in complete medium (CM) or serum-free medium (SFM) for an additional 24 hr, and whole cell lysate were analyzed by Western blot analysis. (B) Cells were transfected with adenovirus expressing TR3 (TR3-Ad) or vector only (10 MOI) at 24 hr post-transfection of siSESN2, and the cells were collected at 48 hr after transfection of TR3-Ad. The cells were incubated in SFM for 24 hr before collection.



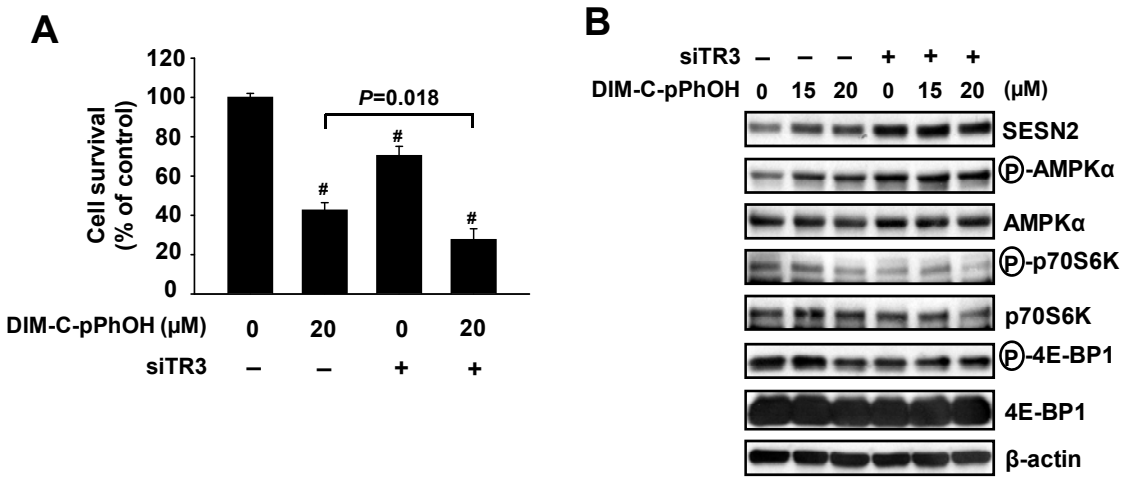
Supplemental Figure 4. Effect of p53 overexpression on mTORC1 signaling in H1299 (p53-null) cells. (A) H1299 cells were transfected with an indicated siRNA for 72 hours. (B) H1299 cells were transfected with 0.2 μg of pCMV-p53-wild type (p53-wt) or pCMV-p53-mt135 mutant (p53-mt135) for 4 hr at 24 hr post-transfection of siScr or siTR3, and the cells were collected at 48 hr after transfection of p53 plasmid DNA. Whole cell lysates were analyzed by Western blot analysis and β-actin was used as a loading control.



Supplemental Figure 5. Association of TR3 with p53 and effect of TR3 overexpression on p53 activity in NSCLC cells. (A) Coimmunoprecipitation. Cells were transfected with Flag-empty or Flag-TR3 for 4 hr, and the cells were collected at 24 hr after transfection. Flag was immunoprecipitated with a Flag antibody and the immunoprecipites were analyzed by Western blots using an anti-p53 antibody. (B) Cells were cotransfected with TR3-Ad and $p53_{x14}$-Luc, and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control, and the results are presented as means with SD. (C) ChIP assay. Cells were transfected with TR3-Ad for 24 hr, and binding of p53 to the SESN2 promoter region containing p53 binding site was determined as described in the Materials and Methods. (D) Cells were cotransfected with TR3-Ad and pSESN2-Luc for 24 hr, and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control, and the results are presented as means with SD.



Supplemental Figure 6. DIM-C-pPhOH induces apoptosis in p53 wild-type and p53-null lung cancer cells through inhibition of p300/Sp1 axis. (A) H460 (p53 wild-type) cells were transfected with each luciferase reporter plasmid as indicated for 5 hr, and treated with DIM-C-pPhOH for 18 hr. Luciferase activity (relative to β-galactosidase activity) was determined, and the corresponding empty vector was used as a control. The results are presented as means with SD of 3 experiments. **P*<0.05, ***P*<0.005, and $^{\#}$*P*<0.001 vs DMSO. (B, left panel) H460 cells were treated with various concentrations of DIM-C-pPhOH for 18 hr, and survivin mRNA level was determined by real-time PCR. TBP was used as an internal control. (B, right panel) H460 cells were transfected with each luciferase reporter plasmid as indicated for 5 hr, and treated with DIM-C-pPhOH for 18 hr. **P*<0.05, ***P*<0.005, and $^{\#}$*P*<0.001 vs DMSO. (C) DNA binding activity of Sp1 or p300 to GC-rich Sp1 consensus sequence. H460 cells were treated with either DMSO or DIM-C-pPhOH for 12 hr, and nuclear extracts were tested for DNA binding activity as described in the Materials and Methods. (D, left panel) H1299 (p53-null) cells were treated with either DMSO or DIM-C-pPhOH for 3 days, and the number of cells in each well was counted on days 1, 2, and 3. (D, right panel) H1299 cells were treated with either DMSO or DIM-C-pPhOH for 24 hr, and whole cell lysates were analyzed by western blot analysis. The experiment was repeated 3 times with similar results.



Supplemental Figure 7. Effects of DIM-C-pPhOH on cell growth and mTORC1 activity in A549 NSCLC cells transfected with siTR3. (A) Cell survival. After transfection with either siScr or siTR3 for 2 days, cells were treated with DIM-C-pPhOH for an additional 2 days. The number of cells in each well was counted and the data are presented as means with SD. $^{\#}P<0.001$ vs siScr without DIM-C-pPhOH. (B) After transfection with either siScr or siTR3 for 2 days, cells were treated with DIM-C-pPhOH for an additional 2 days.